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GONAD: a novel CRISPR/Cas9 genome editing method that does not require ex vivo handling of embryos

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Abstract

Transgenic technologies used for creating a desired genomic change in animals involve three critical steps: isolation of fertilized eggs, microinjection of transgenic DNA into them and their subsequent transfer to recipient females. These *ex vivo* steps have been widely used for over 3 decades and they were also readily adapted for the latest genome editing technologies such as ZFNs, TALENs and CRISPR/Cas9 systems. We recently developed a method called GONAD (Genome editing via Oviductal Nucleic Acids Delivery) that does not require all the three critical steps of transgenesis and therefore relieves the bottlenecks of widely used animal transgenic technologies. Here we provide protocols for the GONAD system.

Keywords

GONAD; Genome editing; microinjection; transgenic; CRISPR/Cas9; gene delivery; *in vivo* electroporation

INTRODUCTION

The genome engineering technologies broadly fall into two categories: transgenic and knock-out (KO)/knock-in (KI). In a transgenic animal, an exogenous gene is transferred into the genome whereas in a KO/KI model, an endogenous gene is deleted (KO) or modified (KI). Typically, the transgenic models are generated by isolating fertilized eggs,

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microinjecting the DNA of interest into them and their subsequent transfer to recipient females. On the other hand, generation of the KO/KI models involve an additional step—by first modifying the wild-type genetic locus through homologous recombination (HR) in embryonic stem (ES) cells: such modified cells are then microinjected into isolated embryos at blastocyst stage followed by transfer of embryos to recipient animals (Behringer et al., 2014).

The recent addition of newer genome editing tools, such as sequence-specific nucleases, has brought many changes to the way with which the long-used genome engineering technologies have been performed. Specifically, because of its simplicity and high efficiency, the CRISPR/Cas9 system has enabled the technology developers to device newer tools that have brought many paradigm shifts to the traditional transgenic protocols. Some examples are: 1) until recently, it was thought that the targeted gene modification always requires the use of ES cells to manipulate the genes through HR; this is not a general rule anymore and targeted gene modification can be achieved through direct injection of DNA into embryos, bypassing the need to use ES cells (Skarnes, 2015), 2) RNA and protein components are also injected into embryos to perform genome engineering (Aida et al., 2015), whereas only DNA was injected previously for production of transgenic models, 3) the pronucleus (or pronuclei), in the zygotes, was the site of injection with the previously used tools, and now cytoplasmic injection is shown to be sufficient for newer tools (Horii et al., 2014), 4) microinjection, as a delivery method, can also be bypassed by using electroporation of the components to the isolated embryos (Kaneko et al., 2014) and finally, 5) a method we recently developed called GONAD can bypass all the three major steps of transgenesis viz., isolation, microinjection and surgical transfer of embryos, and therefore this method relieves the bottlenecks of the long-used animal transgenesis protocols (Takahashi et al., 2015).

The GONAD method essentially delivers the genome editing components directly into the fertilized eggs within oviducts *in situ* (Takahashi et al., 2015). Therefore the method does not require sophisticated micromanipulation systems and also does not require specialized skills in embryo isolation, microinjection, and embryo transfer surgeries. A schematic showing the comparison of steps needed in the traditional gene editing and the GONAD methods is shown in Figure 1.

In this unit, we describe step-by-step protocols of the GONAD method. We would like to add a few general comments. 1) Our group reported this method for the first time; although it is currently being tried in many labs worldwide, at present there are not many collective experiences from other labs in using this method. Therefore, we have made every attempt to include as much detail as we can from our own experiences. 2) Additionally, we have included many images taken while performing the GONAD procedure, which should provide sufficient information and guidance to the researchers. 3) While we used mouse as a model in our system, the method can be tried in other species. Special considerations that may need to be taken into account, in such cases, are described in the commentary section.

At the outset, the GONAD method follows similar steps that were described in UNIT 15.7, “Mouse Genome Editing using the CRISPR/Cas System” (Harms et al., 2014) except that, i)

it bypasses the three major steps; isolation of embryos, microinjection and transfer to recipient animals and a series of sub-steps described in those sections, and ii) those three steps are replaced by one new step that employs an *in situ* electroporation-based delivery of genome editing components to the embryos, within the oviduct. We recommend the reader refer to our previous article (Harms et al., 2014) for the sections that are common (e.g., Basic Protocol 1: designing of CRISPR targets, Basic Protocol 2: synthesis and purification of RNA and DNA components and Basic Protocol 4: genotyping of offspring).

There are five steps in the GONAD protocol:

- I. Preparation of nucleic acids (genome editing components) solution
- II. Preparation of mice for GONAD procedure
- III. Surgical exposure of ovary, oviduct and part of the uterus
- IV. Intraoviductal instillation of nucleic acids
- V. *In vivo* electroporation

MATERIALS

Genome editing components: single guide (sg) RNA and Cas9 mRNA stock solutions, both at about 2 to 4 µg/µl concentration.

0.5% Trypan blue stock solution (#29853-34, Nacalai tesque Inc.)

Phosphate-buffered saline without Ca²⁺ and Mg²⁺ (PBS)

Paper towel pieces*

KimWipe*; Jujo-Kimberly Co. Ltd. * *We suggest using a new box of paper towel and Kimwipes each time to ensure that they are clean and free of any contaminants such as dust and microbes.*

100 mm tissue culture dishes, to use as containers for paper towel pieces and KimWipes

Surgical instruments

- ① No. 5 Forceps (#No.5, INOX)×2, Forceps (#A-7 and A-10, NAPOX, Natsume)
- ② Aorta-Klemme (30-mm in length; #C-17, NAPOX, Natsume)×2
- ③ Scissors (#B-12H, Natsume)
- ④ Suture Wound Clips (MikRon 9mm AUTOCLIP, BECTON DICKINSON)

All surgical instruments should be sterilized by immersing them with 70% ethanol.

Instruments for anesthesia

Bottle/Jar and Disposable syringe (50-ml)

Isoflurane-soaked absorbent cotton balls

20% isoflurane (Escain, Mylan Inc.; usually stored at 4°C) diluted with propylene glycol (#164-04996, Wako) is warmed to room temperature just before use. Approximately 20 ml of 20% isoflurane is needed for 5 mice.

Spray bottle with 70% ethanol

Equipment

Dissecting microscope (e.g., Olympus SZ11) with a hot plate (e.g., KM-1, Kitazato)
 BTX T820 electroporator, with tweezer-type electrodes (made from Nepa Gene Co., Ltd. www.nepagene.jp/)

Mouth pipetting device: This is similar to one used for DNA microinjection into zygotes in a transgenic mouse production experiment. There are many types of mouth pipetting devices used by the transgenic research community, that are either available commercially or assembled using different components (Williams et al., 2011; Liu et al., 2013). The one described here is assembled using a mouth piece, silicon tubing, Pasteur pipette and a glass capillary. A mouth piece is inserted into one end of silicon tubing, and to the other end, a 9" long Pasteur pipette is inserted that has pre-inserted cotton plugs on both ends. The other end of the Pasteur pipette is fitted with a silicon plug [accessories of Microcaps 5 µl (#1-000-0050, Drummond Scientific Company)] into which a pulled-glass capillary is inserted. The glass capillary (#GDC-1, Narishige) is pulled by a micropipette puller (P-97/IVF; Sutter) to make a pointed end (see Figure 4B). The preparation of microinjection needles is performed as described in Harms et al., 2014 with some modifications in needle pulling settings: Glass: # GDC-1; Heat: 790; Pull: 35; Velocity: 50; Pressure: 500; Time: 100.

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There are five steps in the GONAD protocol:

- VI.** Preparation of nucleic acids (genome editing components) solution
- VII.** Preparation of mice for GONAD procedure
- VIII.** Surgical exposure of ovary, oviduct and part of the uterus
- IX.** Intraoviductal instillation of nucleic acids
- X.** *In vivo* electroporation

I. Preparation of nucleic acids (genome editing components) solution

Designing and preparation of sgRNA and Cas9 mRNA have been described in UNIT 15.7, Harms et al., 2014. Note that the GONAD procedure needs about 10 to 100-fold higher concentration of sgRNA and Cas9 mRNA than it is required for microinjection-based methods. The final concentrations of CRISPR components needed for GONAD are 0.5 µg/µl of sgRNA and 1 µg/µl of Cas9 mRNA. Because the procedures described in Harms et al., 2014 generate the concentrations of these components sufficiently high enough (typically about 1 to 3 µg/µl or sometimes up to 4–5 µg/µl), those protocols are also suitable for preparation of CRISPR components for the GONAD method. Once generated, the RNAs can be stored at –80°C as single use aliquots of about 1 to 3 µl per aliquot. On the day of GONAD procedure, one aliquot each of sgRNA and Cas9 mRNA are thawed on ice, mixed and diluted to the desired concentrations. The nucleic acids for GONAD procedure are prepared in nuclease-free water and the trypan blue dye is added to a final concentration of 0.05%, which serves as a visible marker for successful nucleic acids instillation. The considerations for including a repair template, such as single-stranded oligodeoxynucleotides (ssODNs) or plasmid-based double-stranded DNA templates, are described in the commentary section.

II. Preparation of mice for GONAD procedure

All procedures involving laboratory animals should be performed according to institutional and national guidelines and legislation. For each GONAD experiment, we suggest to use about 5–10 pregnant females at 1.5 day of gestation (confirmed for the presence of the vaginal plugs after mating with males) following the standard protocols (Behringer et al., 2014). See commentary section for superovulation consideration.

1. Prepare a set of a jar or a bottle and a syringe for anesthesia. Place cotton balls soaked in ~2 ml of 20% isoflurane, one each in the jar and the syringe as shown in Figure 2D.
2. Wait for about 2~3 min for isoflurane vapors to build up in the jar. During this time, place the nucleic acids solution to the inner wall of a microfuge tube close to the cap as shown in Figure 2E (this is a preparation step needed for step 9).
3. Place a mouse in the anesthesia jar/bottle. Approximately 1~2 min later, when the mouse starts to become anesthetized, transfer the mouse to the syringe containing the isoflurane swab (Figure 2D).
4. Ensure that the mouse is fully anesthetized as examined by toe pinch reflex. If necessary, an additional 1 ml of isoflurane can be added to the cotton swab to ensure full anesthesia. The mouse, along with the isoflurane syringe, is kept on a paper towel placed on the warm plate, which is installed under the dissecting microscope and set at 37°.

III. Surgical exposure of ovary, oviduct and part of the uterus

5. Spray 70% ethanol onto the back skin of the anesthetized mouse. Make a dorsal incision (5~10 mm long; as shown in Figure 3A) at the central portion (marked with an arrow) of the back.

6. Make an incision (spanning 3~5 mm) in the muscle layer. To locate an ovary, grasp the skin with forceps and move the incision either to the left or right side (as shown by the symbol ' $\Leftrightarrow$ ' in Figure 3A). Grasp the adipose tissue surrounding the ovary with the forceps and pull the tissue outside of the incision. Place the exposed ovary, oviduct and part of the uterus on a small piece of paper towel (about 2–3 sq cm) as shown in Figure 3B and anchor the adipose tissue with an Aorta-Klemme in order to prevent return of the exposed tissues (as shown by an arrow in Figure 3B). If bleeding occurs, blood can be removed by gently blotting with small pieces of paper towels.

IV. Intraoviductal instillation of nucleic acids

7. While watching through the microscope, position the ovary, oviduct and uterine tissues, as depicted by a red circle in Figure 2C (see also Figure 3D).
8. The small red circle '○' marked on the oviduct in Figure 4A indicates the point where the nucleic acids solution will be injected. By moving the Aorta-Klemme, position 'this point of the oviduct' to the center of the field of view. Note that this region of the oviduct is relatively whitish (paler) and less fragile than the other parts and therefore this region is better suited for grasping the oviduct with forceps during the instillation step.
9. After positioning the oviduct, draw about 2 μ l of nucleic acids solution into the micropipette controlled by a mouthpiece while watching the droplet (Figure 2E) being drawn under the microscope. Step 2 described above (i.e., placing the solution droplet close to the cap of the tube) will aid in monitoring of the tip of the micropipette while the solution is being drawn into it. Close observation of the tip to ensure proper loading of the solution, and to ensure no damage to it, will be difficult if the solution is loaded directly from the bottom of the tube. Note that at this stage the mouse is positioned and focused under the microscope: in order to watch the solution being drawn under the microscope, hold the tube just above the mouse and adjust the focus to the tube.
10. Holding a No. 5 forceps in one hand, gently grasp the oviduct close to the point marked by the red circle (○) in Figure 4A. With the other hand, insert the micropipette into the lumen of the oviduct by piercing through the oviductal wall. The blue line in Figure 4A indicates the position and direction of the micropipette. The dotted blue line denotes the portion of the micropipette inside the lumen of the oviduct.
11. Inject the nucleic acids solution while holding the oviduct with the No. 5 forceps. The quantity of the solution injected will be sufficient if the trypan blue dye fills the oviduct up to the base of its triangular end (shown by an arrow in Figure 4B; bottom right inset). The direction of the tip should be towards the uterus (i.e., away from the ovary). Once the tip is inserted into the lumen, gently move it back and forth to ensure that the tip is well-inside the lumen. If the tip is incorrectly inserted, the solution may flow towards the ovary (instead of flowing towards the uterus where the embryos are normally present at this stage of

pregnancy). Inserting the micropipette can occasionally cause damage to the oviduct as the oviduct needs to be held firmly with the forceps. Figure 4C shows a correctly instilled oviduct.

12. Withdraw the micropipette after instilling the solution. The left over RNA solution in the pipette can be collected into a tube to check the intactness of RNA by gel electrophoresis.

V. *In vivo* electroporation

13. Immediately after the instillation of nucleic acids into the lumen, cover the oviduct with pieces of wet KimWipe towels soaked in PBS and then place the tissues (oviduct, including the ovary and the adipose tissue) between the two electrodes as shown in Figure 5A. This is a critical step that would require correct placing of two to three pieces of KimWipe towels to prevent injury caused by electric shock and to reduce generation of air bubbles between the electrodes; both of these can result in failure of nucleic acids delivery. It is suggested not to touch the mouse, as a precaution to avoid electric shock, during electroporation, although such shock can be very minor.
14. Apply square pulses (50 V, 5 ms in length, 8 times, with a pulse-off of 1 second) using the BTX T820 electroporator. The delivery of pulses can be confirmed by slight convulsion of mouse itself, and generation of fine bubbles between the oviductal tissues and the electrodes (as observed under the microscope).
15. After electroporation, remove the KimWipe pieces and return the tissues (ovary, oviduct, uterus and adipose tissue) to the original position using regular forceps.
16. Repeat steps 6 to 15 to perform GONAD procedure on the other oviduct.
17. After completing the GONAD procedure on both oviducts, close the incision using Suture Wound Clips (Figure 5B). Keep the mouse on a hot plate (37°C) until awake.
18. Analyze the offspring (embryos at different stages of pregnancy or live born pups, as per your experimental design) for genome editing by various genotyping options described in Harms et al., 2014.

COMMENTARY

Background Information

From over three decades until very recently, the microinjection of embryos has been the unavoidable step in delivering nucleic acids (or mutated ES cells) to create genetically engineered animal models. Newer tools such as site-specific nucleases that were added in recent years to the genetic engineering toolbox enabled researchers to think ‘out of the box’ and try new approaches of delivering the genome editing components to embryos. The first report of electroporation of CRISPR reagents to isolated embryos demonstrated that the microinjection step in animal transgenesis is not absolutely essential. A step beyond this achievement was to bypass the other two critical steps of animal transgenesis—isolation of embryos and transferring of embryos back into recipient females after microinjection. The

GONAD, our recently developed method indeed demonstrated for the first time that animal genome engineering can be accomplished without the need for isolation-, microinjection- and transfer of embryos- back to recipient animals—all three critical steps of animal transgenesis. While the GONAD system can be readily adapted by the mouse transgenic research community, the method offers a valuable tool in other species where embryo handling and manipulation techniques for genome engineering have not been well established. It is anticipated that GONAD-like approaches in other organisms may become established in the near future.

Critical Parameters and Troubleshooting

We discuss here a few critical parameters that are based on our experiences in developing the system (Takahashi et al., 2015).

Concentration of Cas9 mRNA and sgRNAs—Unlike microinjection-based approaches that require very low concentrations of nucleic acids, the electroporation-based procedures require about 10–100 fold higher concentrations of nucleic acids. The requirement for such high concentrations is clearly understandable for reasons such as i) the vast majority of nucleic acids get used up by electroporation into the oviductal tissues rather than their delivery to the embryos, and ii) the delivered nucleic acids should be available near the embryos, at their natural location, in order to ensure enough copies of nucleic acids entering into each embryo when electroporation is applied. If lower concentrations of nucleic acids are injected, such concentrations can reach below the threshold level necessary for their successful delivery into the embryos.

In our report we tried 469 and 530 ng/μl of CRISPR sgRNA and 1096 ng/μl of Cas9 mRNA (with or without 632 ng/μl of eGFP mRNA that served as a marker for successful electroporation, used in experiments where embryos were collected and analyzed before implantation) (Takahashi et al., 2015). Based on this, we think that about 500 ng/μl of sgRNA and 1,000 ng/μl of Cas9 mRNA would be the suggested concentrations for CRISPR/Cas9 components in the GONAD procedure. We have not tested simultaneous delivery of multiple sgRNAs in the GONAD procedure. It may be possible to combine two or more sgRNAs in one GONAD experiment assuming that nucleic acids concentrations can be reached up to about 4–5 μg/μl without facing any solubility or aggregation issues. The fact that all three components we injected (sgRNA, Cas9 mRNA and eGFP mRNA) were delivered effectively to some embryos suggests that at least two separate guides and a Cas9 mRNA (total three components) can be readily delivered using GONAD.

Although it is theoretically possible, we did not attempt creating knock-in mutations by including a repair template in our first report (Takahashi et al., 2015). The ssODNs, serving as repair templates, can be readily synthesized and included at the concentrations as needed. In this case, it is intuitive to include the repair ssODNs at about 500 ng/μl concentration (similar fold increase as other components). It is also theoretically possible to use plasmid-based double-stranded DNAs as templates that contain longer homology arms, similar to those used in microinjection-based experiments. Further, our previous work demonstrated that plasmid DNAs can also be delivered to embryos through *in vivo* electroporation (Sato et

al., 2012). Although we delivered plasmid DNAs via electroporation to oviducts for direct expression at concentrations of 500 ng/μl (Sato et al., 2012), we have not tested the optimal concentration requirements of plasmid DNAs were they to be used as repair templates in CRISPR/Cas9 system.

Time of pregnancy—Timing of pregnancy is important for two reasons: i) accessibility of embryos for efficient flooding of nucleic acids near them, and ii) in reducing possible mosaic mutations.

Accessibility of embryos for efficient flooding of nucleic acids near them: In our report, we tried 1.5 day-old pregnant females, assuming that this would be an optimal time when the majority of embryos (2-cell embryos) would be floating in the oviduct. We presumed that 0.5 days time would probably be a bit early because the embryos would be tightly covered by cumulus cells, which would act as a barrier for reaching an effective concentration of nucleic acids close to them. A speculative-counterargument to this is that we observed a reasonably strong eGFP signal through the oviductal walls, indicating that even if the nucleic acids solution is within the lumen, it may have entered the cells that are farther away from the lumen that are not directly facing the lumen. In the absence of thorough and systematic analyses of eGFP expression in cells located farther away from the lumen, through tissue sectioning and histological analysis, this argument remains highly speculative and future studies may be designed to test this parameter systematically. If future studies indeed observe that the target cells need not be in direct contact with the nucleic acids solution, then it is likely that even 0.5 day-old pregnant females may also be attempted for GONAD.

Reducing possible mosaic mutations: We performed GONAD at 1.5 days of pregnancy, during which time the majority (or all) of the embryos would be at the two-cell stage. One drawback of such a late stage of pregnancy is that it can increase the likelihood of mosaic mutations for reasons such as i) Cas9 not entering into all cells in the embryo (for example, only one cell out of two cells receiving enough Cas9), ii) variable activity of Cas9 in different cells (for example, even if Cas9 enters into all cells, it may not act efficiently in some cells) and iii) different types of mutations created by NHEJ. In our experiments, we noticed both non-mosaic and mosaic offspring. Because all of the embryos will be at the two-cell stage, it is intuitive to think that the chance of getting mosaic mutations is quite high in GONAD. However, we noticed several non-mosaic mutations in our experiments, which could be because of reasons such as i) one of the two blastomeres may have been killed by the electric shock and all cells in the offspring may have derived from the living blastomere or ii) both blastomeres may have been repaired by microhomology-mediated end joining (MMEJ) (Quadros et al., 2015). Therefore, it is likely that performing GONAD on 0.5 day-old pregnant females may reduce the occurrence of mosaic mutations, provided the nucleic acids can efficiently get into cells/tissues that are farther away from the lumen where the nucleic acids will be instilled.

More experiments performed at the timeframe we tried in our first report (~39 to 43 hours post-mating) and additional timeframes (for example, 12 to 18 hours and 18 to 24 hours post-mating) would test these two parameters systematically.

Electroporation conditions—In our first report, we assessed the effect of electroporation on the viability of embryos by isolating and culturing the embryos one day after the procedure from the animals subjected to GONAD (Takahashi et al., 2015). We noticed that approximately half of the embryos looked abnormal and they did not develop properly, whereas most of the control embryos (from animals that were not subjected to GONAD) developed normally. This indicates that although we obtained sufficient numbers of genome-edited offspring, the electroporation conditions that we used could cause significant amounts of embryo death. We think that electroporation conditions may need further optimization to prevent embryo loss while obtaining a high rate of genome-edited offspring. Specifically, optimizing electroporation conditions by varying electric strength and pulse durations may help reduce electrophoretic damage to the embryos. We have successfully used the BTX electroporator for the GONAD procedure and our understanding is that the T820 model BTX electroporator is no longer manufactured and we suggest an equivalent model that will deliver the same pulse.

Site of nucleic acids instillation—While ‘different sites of instillation’ parameters can be tested in larger species such as rabbits, pigs, goats, etc., it may be difficult to optimize this parameter further in mouse because of its small size for delivering the nucleic acids solutions. In this mouse system, fimbriae may be tried as a site for instillation (although we have not yet tested it).

Volume of nucleic acids solution—Typically 0.5 to 1 μ l solution/oviduct is an optimal volume in mouse, as assessed by the filling of the trypan blue dye within the entire oviduct. We suggest drawing at least 2 μ l solution in to the micropipette tip before instillation; occasionally up to 2 μ l solution could be instilled to fill the entire oviduct. Higher volumes may be necessary to achieve the same level of filling in other species. Alternatively, different sites can be attempted in those species (as discussed above) using smaller amounts of solution.

Superovulation—If effective GONAD procedure occurs in an experiment, it is likely that the natural mating is sufficient to generate a few genome-edited offspring. Some factors like damage of the embryos by the electroporation shock and possible flushing of embryos to the edge of the nucleic acids solution (where effective concentrations of nucleic acids in the vicinity of embryos may become diluted) may reduce the overall number of embryos that receive the nucleic acids effectively. A superovulation process may increase the number of embryos but it should be noted that if too many embryos survive the procedure, and if all of them implant, it may add burden to the mother. It would be wise to consider preparing a set of foster females if superovulation is followed, as some superovulated animals occasionally have difficulties in natural delivery.

Additional troubleshooting points—i) Checking the quality of RNA by electrophoresis prior to performing the GONAD procedure is critical, ii) use of proven stud male mice for breeding with females to have enough number of fertilized eggs, iii) if the GONAD procedure does not yield genome edited offspring, even after using the sgRNAs that are proven to work, we advise testing the GONAD procedure by delivering eGFP

mRNA and examining delivery at the morula to blastocyst stage by observing eGFP fluorescence.

Anticipated Results

Some animals subjected to the GONAD procedure may retain pregnancy and some may not. In our hands, we typically obtained at least one pregnant female yielding mutant offspring when about five mice were subjected to the GONAD procedure. The reasons for failure to retain pregnancy in some animals could be i) loss of embryos due to electric shock (and/or excessive flushing), ii) the animals may not be pregnant to start with, and iii) another less likely possibility could be that all embryos rendered homozygous by CRISPR/Cas9 mutagenesis and, if the homozygous mutation is lethal.

The two most critical steps in performing the GONAD procedure are i) the ability to grasp and hold the oviductal tissue firmly but gently during the process and ii) the ability to successfully instill nucleic acids solution into the oviductal lumen. Therefore, success in establishing the GONAD technique in a new lab depends on the skill levels of the person performing the technique. A person proficient in traditional transgenic technologies with extensive experience in handling oviducts, such as oviductal embryo transfer techniques, can easily learn the GONAD procedure and would successfully generate mutant offspring in their first session from about 5 to 10 pregnant mice subjected to the GONAD procedure. For a person with limited experience in transgenic technologies, it is advisable to use at least 10 or more pregnant mice per session and it may take about 2 or 3 sessions before successfully establishing the technique.

Time Considerations

A typical timeframe for the GONAD method is outlined below:

Weeks 1 – 3:	Designing and synthesis of genome editing components and genotyping primers.
Weeks 2 – 3:	Procuring of animals and testing genotyping primers on wild-type genomic DNA.
Weeks 4 – 5:	GONAD procedure. Note that the entire GONAD procedure can take about 10 to 20 min per animal.
Weeks 8 – 10:	Genotyping of offspring.

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Figure 1.
Comparison of the protocol-steps in the traditional gene editing and the GONAD methods

Figure 2.

Equipment and instruments needed for GONAD. (A) Arrangement of equipment (electroporator with electrodes, dissection microscope with a hot plate) and surgical instruments needed for the GONAD procedure. (B) Higher magnification of electrodes and the schematic of the platinum concave electrodes. (C) List of surgical instruments: ① Forceps, ② Aorta-Klemme, ③ Scissors, ④ Suture Wound Clips. (D) Anesthesia equipment: Anesthesia bottle/Jar (left), Disposable 50 ml syringe (right top) and the anesthetized mouse from the jar transferred to the syringe photographed just prior to surgery. (E) Microcentrifuge tube containing the nucleic acids solution droplets placed close to the cap (left), loading of the nucleic acids solution (middle) and a schematic of droplet drawn into the needle-tip (right). (F) Paper towels (kept ready if necessary) and KimWipe pieces soaked in PBS needed during electroporation.

Figure 3.
Surgical exposure of oviducts for GONAD procedure: See text for details (steps 5 to 7)

Figure 4.

Intraoviductal instillation of nucleic acids. (A) Higher magnification of the exposed tissues showing the point of instillation in a red circle, and schematic of the instillation needle is shown as a dotted line from the point of instillation. The inset shows the Aorta-Klemme holding the tissues firmly for instillation. (B) Shown are the various parts of the mouth pipetting device. The insets show higher magnification of the part of Pasteur pipette and the instillation needle filled with nucleic acids solution with trypan blue dye (see text for details of assembling mouth pipetting device). (C) Picture of the oviduct showing the correctly instilled nucleic acids solution.

Figure 5.

Electroporation. (A). Photograph of the tissues held between the electrodes showing the oviduct instilled with nucleic acids solution and covered with 2 to 3 layers of pre-cut KimWipe towels soaked in PBS (see step # 13 in text for more details). Note that even though the bottom electrode is not in close contact with the oviductal tissue (when this picture was taken), both the electrodes (marked with red asterisks) should be in close contact with the oviductal tissue during electroporation. (B) Mouse after completion of the GONAD procedure.